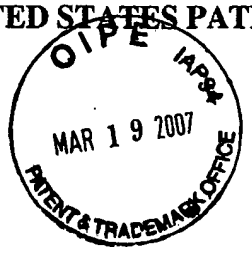
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



In re Application of:
HEATH, *et al.*
Serial No.: 08/699,716
Filed: 27 August 1996
For: RECOMBINANT F1-V PLAGUE VACCINE

Art Unit: 1645
Examiner: Duffy, Patricia Ann
Atty. Dckt: 003/029/SAP

AFFIDAVIT OF ARTHUR M. FRIEDLANDER

1. I, Arthur M. Friedlander, an inventor of the above-referenced application and resident of Gaithersburg, MD, declare the following:
2. My curriculum vitae is attached.
3. David G. Heath, George W. Anderson, Jr., Susan L. Welkos and I are joint inventors of the subject matter disclosed in the above-referenced application.
4. From 1992 to 1998, I was in charge of the development of a plague vaccine.
5. Sometime during the second or third week of **[redacted date which is before 13 March 1996]**, but before **[redacted date which is before 13 March 1996]**, during an Army Plague Vaccine Group weekly meeting, I decided that we should make a fusion protein comprising the F1 and V antigens (F1-V fusion) of *Yersinia pestis* for use as a new plague vaccine. I asked for a volunteer and David G. Heath volunteered. This is evidenced by Heath's laboratory notebook page dated **[redacted date which is before 13 March 1996]**, page 44 of notebook #3487 which shows the primers and PCR plan to create the fusion protein. See Exhibit AF1.
6. From **[redacted date which is before 13 March 1996]** to the time of filing the above-referenced application with the U.S. Patent & Trademark Office, we conducted research and development of a plague vaccine comprising an F1-V fusion protein.
7. Since about the beginning of **[redacted date which is before 13 March 1996]** to about the filing date of the above-referenced application, members of the Army Plague Vaccine Group of the U.S. Army Medical Research and Materiel Command (USAMRMC) have been collaborating with the inventors of the Titball Patent (US 5,985,285) on a variety of plague vaccines. This is evidenced by Welkos' Army Plague Meeting Notes dated **[redacted date which is before 13 March 1996]**. See Exhibit AF2 (SW3). This is also evidenced by an excerpt of a letter from CBDE which was faxed to me on **[redacted date which is before 13 March 1996]** and catalogued in Anderson's notebook #3598. See Exhibit AF3.
8. Prior to 21 May 1995, the Army Plague Vaccine Group developed and purified a fusion protein containing all of the F1 antigen fused to part of the V antigen (F1-V partial) and a fusion protein containing all of the F1 antigen fused to all of the V antigen (F1-V whole). Since we observed that F1-V partial confers protection against wild-type *Yersinia pestis*, but does not provide protection against F1$^-$ mutant *Yersinia pestis*, we then decided to determine whether F1-V whole confers protection against F1$^-$ mutant *Yersinia pestis*. This is evidenced by Welkos' Army Plague Vaccine Group meeting notes dated **[redacted date which is before**

13 March 1996]. See Exhibit AF4 (SW4).

9. On about 21-25 May 1995, I attended the American Society of Microbiology Meeting in Washington, DC, where I met with Richard Titball of CBDE. When I met with Richard Titball, I told him in confidence that the Army Plague Vaccine Group had constructed and purified the F1-V whole and believe that it will confer protection against F1⁻ mutant *Yersinia pestis*. I noted this discussion in the Invention Disclosure I prepared and submitted on 16 April 1996 to the Office of Research and Technology Applications (ORTA) of USAMRMC. See Exhibit AF5.

10. Some time before 16 April 1996, I compiled the research and data of the Army Plague Vaccine Group relating to F1-V fusion proteins and prepared and submitted an Invention Disclosure. See Exhibit AF5.

11. Shortly after the Invention Disclosure was forwarded from ORTA to the outside contract attorney, Sana Pratt, I began working with Sana Pratt to prepare the application which was filed on 27 August 1996.

12. From just prior to 13 March 1996 to 27 August 1996, David G. Heath, George W. Anderson, Jr., Susan L. Welkos and I did not abandon, suppress or conceal the invention as disclosed and claimed in the above-referenced application.

13. I have reviewed and analyzed the Titball patent and the three priority documents, UK 9505059, UK 9518946, and UK 9524825, and PCT/GB96/00571.

14. It is my opinion that prior to 13 March 1996, the filing date of PCT/GB96/00571, the inventors of the Titball patent had not conceived and/or reduced to practice a plague vaccine comprising purified F1 antigen fused to all or part of V antigen as nowhere do UK 9505059, UK 9518946, and UK 9524825 disclose isolating or purifying a protein comprising F1 antigen fused to all or part of V antigen from the host cell and other cellular components and/or administering a purified protein comprising F1 antigen fused to all or part of V antigen to a subject.

    a. In fact, UK 9518946 is the first disclosure indicating a genetic vaccine or how a host organism may be transfected with DNA for F1 antigen and V antigen to result in a live vaccine, i.e. an attenuated host organism (such as Salmonella) which produces the antigen when administered to a subject.

    b. As described in UK 9518946, the genetic vaccine or the live vaccine is administered to a subject such that the protein/antigen of interest is then produced in the subject.

    c. UK 9518946 does not describe isolating the protein/antigen of interest from the host organism and purifying the protein/antigen of interest from other cellular components prior to administration to a subject.

    d. The genetic vaccine or live vaccine described in UK 9518946 is not a purified protein comprising F1 antigen fused to all or part of V antigen which is isolated and purified from cells and other cellular components as claimed in the above-referenced application.

15. I have reviewed and analyzed the experiments and data of the Army Plague Vaccine Group and it is my opinion that the Army Plague Vaccine Group:

a. Conceived of a fusion protein comprising F1 antigen fused to part of V by at least **[redacted date which is before 13 March 1996]**.

b. Conceived of a fusion protein comprising F1 antigen fused to all of V by at least **[redacted date which is before 13 March 1996]**.

c. Conceived of and reduced to practice a purified fusion protein comprising F1 antigen fused to part of V by at least **[redacted date which is before 13 March 1996]**.

d. Conceived of and reduced to practice a purified fusion protein comprising F1 antigen fused to all of V by at least **[redacted date which is before 13 March 1996]**.

e. Conceived of and reduced to practice a vaccine against plague comprising a purified fusion protein comprising F1 antigen fused to part of V by at least **[redacted date which is before 13 March 1996]**.

f. Conceived of and reduced to practice a vaccine against plague comprising a purified fusion protein comprising F1 antigen fused to all of V by at least **[redacted date which is before 13 March 1996]**.

16. I declare that all statements made herein of my own knowledge are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Arthur M. Friedlander

Date: 15 March 2007

Exhibit AF1

REDACTED

Pick single colony isolated of Antigua CCR-6VH-pBlue B/E and of Barbados CCR-6VH pBlue B/E (2 of each)

- grew up 15 ml cultures ON - will have Sam make Qiagen plasmid DNA & sequence using internal & universal primers or perhaps just universal primers

REDACTED

Rabbits were bled by VMD personnel. Work serum and stored @ 4°C.

Designed primers to segment part of V to F1 capsule had Kristin make 4 primers:

BAMHIF1 5' 5' GAA AAA GAA TCA CAG GAT CGT TTC 3'

EcoRIF1Rev 5' CTC GAA TTC TTG GTT AGA TAC GGT 3'

V168For 5' CAC GAA TTC TCA GTT ATT CAA GCC G 3'

V275REV 5' GTG GGT CGA CTC AAT CCG AGC AGG TGG T 3'

Basic plan is to PCR pYPR1 to get F1 operon from BAMHI site to very end of F1 capsule gene

B——E

for V have from 168 to 275 amino acids

Eco
|
168 —— Sal I & stop codon

ligate the 2 PCR products after B/E & E/Sal digestion to get a B/Sal fragment. Then ligate into B/Sal of pBluescript

B Sal

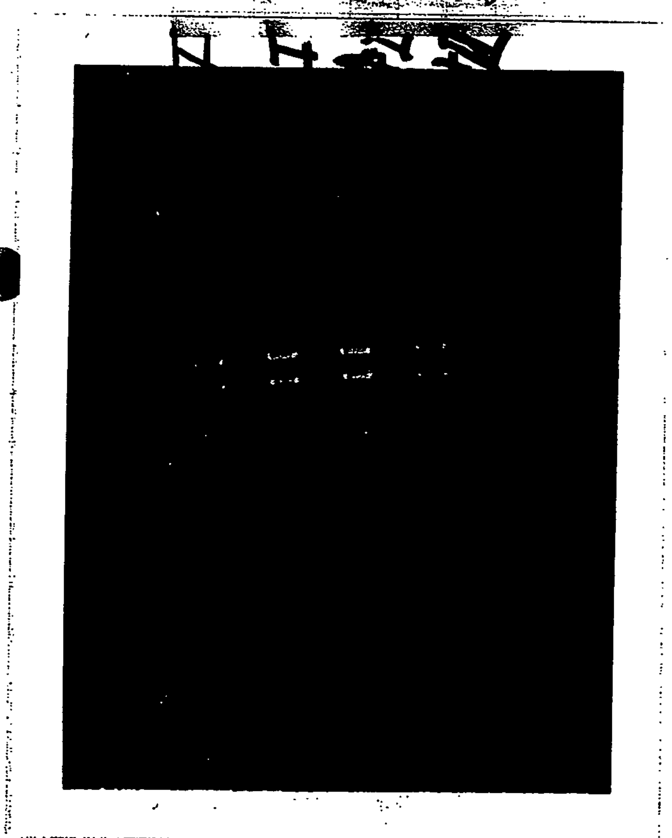
- then ligate new B/Sal piece to E/S small fragment of pYPR1

B —— Sal (EHV) + E —— P (II) = E —— Sal (B)

so now ligate into E —— Sal digested p Bluescript

& grow at 30 or 37°C

REDACTED Had some do Qiagen Plasmid isolation of 4 recombinants c LCR-6VH operon. We then digested c E/B & ran on agarose. We also sequenced insert using reverse & forward primers



Results: The four isolates look like they have about the right sized insert ~2.1KB.

REDACTED set up Ouchterlony using rabbit sera vs. F1

rabbit #1 ○ ○ Anti plaque rabbit

○ F1 40% ext

rabbit #2 ○

using 4 week post injection sera (also 1 boost @ 2nd week)

Rabbit # 1 = 7F7D1D3A12
Rabbit # 2 = 7F7D1D716A

Exhibit AF2

Col. T.'s Priorities = [A] ① "a timeline algorithm"

② Get info from Cutter vaccine - as much as possible.

"Use Cutter in all appropriate protective scenarios"

"Augment with antibiotics."

Col T. being asked:

"Does ~~cipro~~ doxycycline & ciprofloxacin, etc. protect"

Why are we doing FI if British are also?

duplication

We need to meet with Brits to define collaboration

ex. "We need to press & be aggressive."

Definition of the plague ~~threat~~ threat & how are they.

He wants a Brit to come here or one of us to go there to work in lab.

He wants the Brit. & U.S. scientists to get together.

Exhibit AF3

Dear Dr Friedlander

CBDE/USAMRIID COLLABORATIVE RESEARCH INTO PROTECTIVE EFFICACY OF RECOMBINANT V-ANTIGEN AGAINST PARENTERAL AND AEROSOL CHALLENGE WITH YERSINIA PESTIS

As you are aware, CBDE has data to suggest that the V-antigen of the plague causing organism *Yersinia pestis*, when used as an immimogen, is highly protective against plague. The V-antigen could therefore be a major component of an improved plague vaccine to be developed in the future by CBDE.

You recently indicated to us that USAMRIID wished to collaborate in testing the protective capacity of the V-antigen against parenteral and aerosol challenge with virulent plague. We agreed that such a collaboration would be desirable because it could generate valuable data which would be of benefit to both CBDE and USAMRIID. We therefore decided that the collaboration should, in the future, be the subject of a Project Arrangement under the Memorandum of Understanding between the Secretary of Defense (US) and the Secretary of State for Defence (UK) concerning Technology Research and Development Projects (which is currently still under negotiation).

However, we also agreed that any delay in the collaboration would reduce the benefit of the resulting data, and therefore it would be desirable to commence work in advance of a more formal Project Arrangement.

Accordingly, this letter sets out below the respective duties, rights and responsibilities of each of us under the collaboration, *pro tem*, pending the negotiation of a more comprehensive arrangement:

1. SCOPE OF WORK

a. CBDE will supply to USAMRIID, for the purposes described in (b), the following:

i. 30 mg of recombinant *Yersinia pestis* V-antigen.

ii. Protocols detailing the immunisation route, doses and schedules used at CBDE.

iii. Polyclonal antisera raised against the V antigen of *Yersinia pestis*.

iv. Details of the CBDE challenge route, challenge strain and protection data afforded by the V-antigen vaccine against parenteral challenge with *Yersinia pestis*.

b. USAMRIID will:

i. Immunise groups of animals parenterally with the following:

- V-antigen in combination with Alhydrogel.

REDACTED

In-House Plague Review

Exhibit AF4

① Anderson, George

F1 encapsulation vaccine exp. Kenda's carried 3"

(A) 1 shot immunization

48 days challenge (at d. 44)

DOX:

10 μg F1 in microcapsule + 10 μg F1 is "free" (gave higher anti-F1 titers)

SC challenge vs. 67 LD50's

good protection

sterile immunity in spleens at 28 days post challenge

(B) Aerosol Challenge w/ increased dose

1 shot F1 alone - poor

" " " encapsulated - not help

1 vs. 2 doses F1 + alhydrogel → much better w/ 2 doses

∴ no clear cut evidence that microencapsulation ~~not~~ helps vs. aerosol challenge

2. Collaborative work w/ British (CBE)

∴ V Antigen is v. good vaccine

Recombinant V Ag from British ~~[redacted]~~

10 μg: days 0 & 30 → with Alhydrogel ~~[redacted]~~

challenge: day 58 Alhydrogel

C092: ① 61 → 10^6 LD50s challenge SC ---- good survival

② Aerosol - good protection again! [at d. 28!]

59-971 LD50s challenge

all sterile spleens 28 days post challenge

C12: ∴ excellent protection, SC challenge

aerosol challenge --- does not as high, not given as well as C092"

7/8 - low dose challenge: 84 LD50

9/10 - high dose " : 193 LD50

Problem did low immunization for Ab titers

Exhibit AF5

DISCLOSURE RECEIVED AT LEGAL DATE: _____
FROM ORTA: 16 APR 96
LOGGED IN BY: _____
LOG NUMBER: RUD 9608

REDACTED

APPENDIX A

DEPARTMENT OF THE ARMY
UNITED STATES OF AMERICA

INVENTION DISCLOSURE

(THIS FORM AND ACCOMPANYING DRAWING AND DESCRIPTION SHEETS ARE TO BE COMPLETED FOR EACH INVENTION PROMPTLY FORWARDED TO THE PATENT ACTIVITY(8))

PATENT ACTIVITIES DOCKET NO. 16-08

ASSIGNED TO:

SHORT TITLE OF INVENTION
Recombinant F1-V Plaque Vaccine

FULL NAME(S) OF INVENTOR(S) (FIRST)	(MIDDLE INITIAL)	(LAST)	HOME ADDRESS(ES)	(DUTY) TEL. NO. AREA CODE
(1) David	G.	Heath	Bacteriology Division	(301) 619-7341
(2) Arthur	M.	Friedlander	USAMRIID, Bldg. 1425	7341
(3) George	W.	Anderson	Fort Detrick	4433
Susan	L.	Welkos	Frederick, MD 21702-5011	4430

INFORMATION AND DATES CONCERNING THIS INVENTION

NEEDED IN THE EVENT OF A CONTEST OF PRIORITY OF INVENTION IN THE U.S. PATENT AND TRADEMARK OFFICE. ALL RECORDS CITED SHOULD BE DATED AND SIGNED BY TWO INDEPENDENT WITNESSES WHO HAVE READ AND UNDERSTOOD THE MATERIAL.

(4) ON WHAT DATE DID YOU FIRST THINK OF THIS INVENTION: REDACTED (WHAT RECORDS SHOW THIS?) Laboratory Notebook

(5) GIVE DATE OF AND IDENTIFY EARLIEST SKETCH OR DRAWING: REDACTED

(6) WHEN/WHERE AND TO WHOM DID YOU MAKE THE FIRST DISCLOSURE TO OTHERS OF THE INVENTION EITHER ORALLY OR IN WRITING? See enclosure

(7) DESCRIBE DETAILS OF ANY WORK OR TESTS DONE TO PRODUCE OR OPERATE THE INVENTION GIVE DATES AND REFERENCE OTHER PAGES IF NECESSARY): REDACTED

(8) DESCRIBE AND GIVE DATES OF ANY OTHER SKETCHES, DRAWINGS OR REPORTS PERTINENT TO THIS INVENTION

USE, SALE OR PUBLICATION

NEEDED TO ESTABLISH THE DATE OF ANY PRINTED PUBLICATION, PUBLIC USE OR SALE. SINCE NO PATENT APPLICATION MAY BE FILED AFTER ONE YEAR FROM SUCH DATE.

(9) IF INVENTION HAS BEEN SOLD OR USED FOR PROFIT- WHEN AND TO WHOM DISCLOSED OR WHEN AND HOW USED? Not sold or used for profit

(10) HAS A DESCRIPTION OF THIS INVENTION BEEN MADE AVAILABLE TO PERSONS OUTSIDE THE ARMY? (WRITTEN OR ORAL) IF SO, HOW AND WHEN AND WAS USE RESTRICTED? See enclosure

POTENTIAL MARKET INFORMATION

NEEDED FOR POSSIBLE MARKETING INVESTIGATIONS AND AS AN AID TO POTENTIAL LICENSING TO OTHERS.

(11) DESCRIBE ANY POTENTIAL OR EXISTING MARKET FOR SALE OR LICENSE OF THIS INVENTION

A. GOVERNMENT: Vaccine against plague

B. COMMERCIAL: Vaccine against plague

C. IDENTIFY ANY KNOWN FIRMS OR VENDORS WHO MAY BE INTERESTED IN THE INVENTION
Greer Laboratories, Lenoir, NC

CONTRACT INFORMATION

A DETERMINATION OF RIGHTS IN THIS INVENTION WILL BE NECESSARY. (SEE AR 27-60)

(12) IF THIS INVENTION WAS FIRST CONCEIVED OR CONSTRUCTED IN CONNECTION WITH:

(A) MY DUTIES AS A GOVERNMENT EMPLOYEE

B. MY WORK UNRELATED TO MY DUTIES AS A GOVERNMENT EMPLOYEE (PRIVATE, OFF DUTY ACTIVITIES)

C. MY DUTIES AS A GOVERNMENT EMPLOYEE & WORKING WITH A CONTRACTOR

D. NEITHER A, B OR C, EXPLAIN

FOREIGN FILING CONSIDERATION

NEEDED TO DETERMINE THE POTENTIAL WORLDWIDE USE FOR THE INVENTION.

(13) INDICATE THE POTENTIAL FOR USING THIS INVENTION IN FOREIGN COUNTRIES

☐ POOR

☐ GOOD

☒ EXCELLENT

SECURITY CLASSIFICATION

(13A) PLEASE INDICATE THE SECURITY CLASSIFICATION IF KNOWN

☐ CLASSIFIED LEVEL

☒ UNCLASSIFIED

☐ CLASSIFICATION UNKNOWN

DEPARTMENT OF THE ARMY
UNITED STATES OF AMERICA

INVENTION DISCLOSURE

(DRAWING AND DESCRIPTION SHEET)

PATENT ACTIVITIES	DOCKET NO.

(14) PROVIDE THE FOLLOWING INFORMATION CONCERNING THE DISCLOSED INVENTION AND IN THE INDICATED SEQUENCE:

A. SPECIFICALLY DESCRIBE THE INVENTION AND ITS OPERATION. YOU MAY USE AND ATTACH COPIES OF SKETCHES, PRINTS, PHOTOGRAPHS, PAPERS AND ILLUSTRATIONS, WHICH SHOULD BE SIGNED, WITNESSED AND DATED. USE NUMBERS AND DESCRIPTIVE NAMES IN DESCRIPTIONS AND DRAWINGS.

B. STATE THE ADVANTAGES OF THE INVENTION OVER PRESENTLY KNOWN DEVICES, SYSTEMS OR PROCESSES.

C. DISCUSS THE PROBLEMS WHICH THE INVENTION IS DESIGNED TO SOLVE, REFERRING TO ANY PRIOR INVENTION OF A SIMILAR NATURE WITH WHICH YOU MAY BE FAMILIAR.

D. LIST ALL KNOWN AND OTHER POSSIBLE USES FOR THE INVENTION.

E. LIST THE FEATURES OF THE INVENTION THAT ARE BELIEVED TO BE NOVEL.

USE AS MANY OF THESE SHEETS AS NECESSARY AND ATTACH TO COMPLETED INVENTION DISCLOSURE

See attached enclosure

SIGNATURE(S) AND ORGANIZATION OF INVENTOR(S) (USE INK)	DATE:	THE DESCRIBED INVENTION HAS BEEN WITNESSED, READ, AND UNDERSTOOD BY:	DATE:
(15) _____ ORGANIZATION USAMRIID		(18) _____	
(16) _____ ORGANIZATION USAMRIID		(19) _____	
(17) _____ ORGANIZATION USAMRIID		(20) _____	

(18) John J Wilson
USAMRIID

* NOTE: THIS FORM AND ANY OMITTED INFORMATION BECOMING AVAILABLE AT A LATER TIME SHOULD BE FORWARDED TO:

HQDA CHIEF, INTELLECTUAL PROPETY DIV. OFFICE OF THE JUDGE ADVOCATE GENERAL DEPT. OF THE ARMY WASHINGTON, D.C. 20310 DARCOM ATTN: PATENT COUNSEL; OR CHIEF OF ENGINEERS ATTN: PATENT COUNSEL

Invention Disclosure: Recombinant F1-V plague vaccine

(6) First disclosure was in a conversation with Richard Titball, CBDE, Porton Down, England during the American Soc. of Microbiology Meeting sometime during 21-25 May, 1995 in Washington, DC

(10)

a. oral communication in confidence to Richard Titball, American Soc. of Microbiology Meeting sometime during 21-25 May, 1995 in Washington, DC

b. approximately 1/15/96, written abstract sent to Tom Schwan, Rocky Mountain Labs, Hamilton, MT; Kathleen McDonough, David Axelrod Institute, Albany, NY; Dorothy Pierson, University of Colorado, Denver, CO in confidence for review of Army Plague Research program

c. written communication 1/24/96 submitted to the journal Nature

d. oral presentation on 2/15/96 at review of Army Plague Research program, Frederick, MD

e. written communication 3/19/96 submitted to the journal Science

(14)

A recombinant *Yersinia pestis*-derived F1 capsule and V antigen fusion protein.

A. The invention is a fusion protein made up of two proteins derived from *Yersinia pestis*: the F1 capsule antigen (F1) and the V antigen. The process of constructing the fusion protein required several intermediate steps. The first step called for creating a polymerase chain reaction (PCR) product consisting of part of the F1 operon and the F1 structural gene (*caf1*) open reading frame (ORF) from which the stop codon was removed (Figure 1A). The *Bam* HI/*Eco* RI restricted, F1-containing, PCR product was then ligated into the smaller isolated *Eco* RI/*Bam* HI fragment of pYPRI to create pF1LZ (Figure 1B). Next, a small internal segment of the V antigen ORF (Figure 1C) was generated by PCR and ligated into the *Eco* RI (partial digest) and *Sal* I digested pF1LZ to create pF1V3a (Figure 1D). pF1V3a then served as the template DNA in a PCR reaction to create a PCR product containing the F1 structural gene ORF fused, in frame, with the

internal V segment. This PCR product was restricted with *Nde* I and *Bam* HI and ligated into pET19b (Novagen, Inc.) to create pF1Vs (Figure 1E). The V segment and the small *Bam* HI/*Pst* I fragment from the original plasmid vector, pET19b were removed from pF1Vs (Figure 2A) and replaced with the entire V antigen ORF (Figure 2 B) in a ligation reaction (Figure 2C) which also included the small *Bam* HI/*Pst* I fragment from pET19b to create pF1V (Figure 2D). pF1V DNA was used to transform *Escherichia coli* strain BLR (Novagen) and expression of the fusion protein was then shown to occur upon induction with isopropylthio-β-galactoside (IPTG). Expression of a protein of the appropriate size for this fusion protein (58 kDa) was demonstrated by SDS-polyacrylamide gel electrophoresis (Figure 3A). The invention has been designated F1-V.

1. To purify the F1-V fusion protein, *E. coli* strain BLR containing the plasmid pF1V was grown overnight in a small shaking flask using 5 ml of LB broth containing 100 µg/ml of carbenicillin. The overnight culture was then centrifuged at 5000 x g to pellet the cells and resuspended in fresh LB/carbenicillin. One ml of the fresh suspension was used to inoculate 1 liter of LB/carbenicillin and the culture was rotated at 225 rpm and allowed to grow at 37°C for 4 to 5 hr (OD_{600} = 1). The temperature was then lowered to 26°C and IPTG was added to 1 mM final concentration at which time the culture was allowed to rotate at 225 rpm for an additional 2 hr. The cell culture was then centrifuged and cell pellets were resuspended in 40 ml of 1 x Binding Buffer (Novagen, Inc.). The suspension was then subjected to sonication (six 30 sec bursts) and the cell debris was removed by high-speed centrifugation (39,000 x g) for 20 minutes. The supernatant was removed and subjected to ultrafiltration (0.45µ filter) after which it was divided into 10 ml aliquots for storage at - 70°C. The frozen supernatant was allowed to thaw on ice and subjected to fast protein liquid chromatography (FPLC) using a Ni^{2+} chelation resin (Novagen, Inc.). The bound fusion protein was released from the resin after exposure to an imidazole gradient and fractions containing the fusion protein were pooled and buffer exchanged, by dialysis, into 20mM Tris, pH 7.6, 0.5 mM EDTA. The pooled protein was then subjected to a further round of FPLC (to remove endotoxin) using a Mono Q (Pharmacia) ion-exchange column. The purified fusion protein was then tested for endotoxin content using the Limulus amebocyte lysate assay (Sigma). The purified F1-V purified protein was subsequently shown to bind antibody

directed against either the F1 antigen or antibody directed against V antigen (Figure 3b, 3c).

2. The value of the F1-V protein was demonstrated by its ability to protect experimental animals against infection with *Yersinia pestis*, the causative agent of plague. Most forms of naturally occurring plague are due to F1 capsule containing (F1+) strains of *Y. pestis.* However, F1- or deficient plague strains have been isolated from natural sources and from a human case, and are virulent in experimental infections of mice and non-human primates.

In two separate experiments (Table 1), mice immunized with 13.6 μg of F1-V were protected (90-100% survival) against a subcutaneous challenge with a moderate (57 LD_{50}) or high (1.1×10^6 LD_{50}) dose of an F1- *Y. pestis* strain, C12, while all control animals died. Animals given 10 μg of V (equivalent to the same amount of V as in 13.6 μg of F1-V) were afforded the same degree of protection (90% survival) against the high-dose challenge. Another group of animals immunized with 27.2 μg of F1-V completely (100%) survived the high-dose challenge. In a separate experiment (Table 3), animals given just one immunization of 60 μg of F1-V were completely protected against subcutaneous challenge with a high dose of C12, while the licensed human vaccine gave no protection.

We next determined the efficacy of F1-V against pneumonic plague induced by an aerosol challenge (Table 2). Groups of mice immunized with 13.6 or 27.2 μg of F1-V were completely protected (100% survival) against a moderate (91 LD_{50}) or high (545-636 LD_{50}) aerosol challenge dose of the F1- *Y. pestis* strain, C12. In marked contrast, the current human, whole-cell plague vaccine USP, failed to prevent fatal pneumonic plague; none of eight challenged animals survived.

We next determined the efficacy of the F1-V protein in protecting mice against infection with plague strains containing the F1 capsule. Table 3 shows that a single dose of 60 ug of F1-V completely protected mice against a subcutaneous challenge with the F1+ CO92 strain. In contrast the licensed human vaccine protected only 4 of 10 animals. Moreover, while previous data showed that 2 doses of the current human plague vaccine significantly protects mice against a subcutaneous challenge with CO92, it does not protect animals after an aerosol challenge but just delays the time to death (Pitt et al. 1994 Annual Meeting, Amer. Soc. Micro. Abstract #E45, Las Vegas, NV). Furthermore,

the same study showed that the licensed vaccine did not even delay the time to death in non-human primates exposed by aerosol to CO92. Table 2 shows that immunization with 2 doses of F1-V completely protects animals against an aerosol challenge with an F1+ *Y. pestis* strain, CO92, with 10 of 10 animals surviving. Thus the F1-V vaccine, in contrast to the current licensed vaccine, protects mice against pneumonic plague with both F1+ and F1-*Y. pestis* strains, a more difficult form of the disease to protect against.

B. The invention was designed to be used in a vaccine affording protection against plague, due to exposure to the infectious agent *Yersinia pestis.* The advantages of using this fusion protein over the present whole cell vaccine are as follows:

1. The current licensed vaccine does not protect mice against subcutaneous challenge with F1- strains of *Y. pestis*, which have been shown to cause fatal disease in both humans and experimental animals infected by a peripheral, non-respiratory route. The new F1-V vaccine does protect mice against bubonic plague caused by subcutaneous challenge with F1- organisms.
2. The current licensed vaccine does not protect mice against pneumonic plague induced by aerosol challenge with F1- strains of *Y. pestis.* The new F1-V vaccine does protect mice against pneumonic plague caused by aerosol challenge with F1- strains.
3. The current licensed vaccine does not protect mice against pneumonic plague when challenged by the respiratory route with F1+ strains of *Y. pestis.* The new F1-V vaccine does protect mice against pneumonic plague caused by aerosol challenge with F1+ strains.
4. The new F1-V vaccine is expected to protect humans against pneumonic plague produced by strains of *Y. pestis*, either naturally occurring or genetically engineered, which may be altered in their content or composition of V antigen, but which still contain F1. This is because the F1-V vaccine also contains F1. The current licensed vaccine does not protect against pneumonic plague induced by either F1- or F1+ organisms when given by the aerosol route.
5. The new F1-V vaccine is composed of two antigens, both of which have been shown to be protective. It is anticipated that the combination of both antigens may provide better protection against F1+ strains than

either F1 or V when used alone as vaccines. This is possible because the immunity induced by F1 and by V occur by different mechanisms which may be additive or synergistic.

6. Approximately 8% of humans immunized with the current licensed human plague vaccine fail to develop an immune response to F1 (Marshall et al. J. Inf. Dis. 129:S26-S29, 1974). These non-responders may well be at risk for development of plague. The inclusion of two different protective antigens in the same vaccine will help to eliminate the problem of non-responders and so increase the overall efficacy of vaccination in a human population.

7. The new F1-V vaccine is composed of highly purified recombinant proteins which are very well defined. This contrasts with the present human licensed vaccine composed of whole bacteria. The nature of the protective immunogen(s) in the present vaccine is completely unknown. The present vaccine in known to contain and induce antibodies to F1 but it does not induce antibodies to V antigen in mice, suggesting that V antigen is absent. Furthermore, it is anticipated that the highly purified new F1-V vaccine will be significantly less reactogenic in humans than the present human licensed vaccine, which may contain unnecessary bacterial components responsible for its untoward side effects.

8. The F1-V protein was constructed so that a single protein could be purified as a vaccine component rather than having to purify F1 and V antigen separately. The purification of a single protein as opposed to two separate proteins could result in considerable savings when manufacturing a vaccine.

C. This invention is designed to solve the problem of protecting humans against both bubonic and pneumonic forms of plague caused by infection by the subcutaneous and aerosol routes, respectively, with either F1+ or F1- plague organisms, or with strains which may vary in their V antigen.

The current licensed human vaccine protects mice against subcutaneous challenge with F1+ strains, but only delays the time to death of mice challenged by the aerosol route. The vaccine has no protective effect and does not delay the time to death in the non-human primate exposed to F1+ organisms.

The current licensed human vaccine has no significant effect on survival of mice challenged with the F1- C12 strain by either the subcutaneous or the aerosol route.

Thus the current licensed human vaccine would be expected to be ineffective against pneumonic plague caused by either F1+ or F1- strains, or bubonic plague produced by F1- strains of *Y. pestis.*

D. Known or possible uses of this invention include the following: 1) The fusion protein could be used as a vaccine to protect against bubonic or pneumonic plague due to both F1+ and F1- strains of *Y. pestis* or strains which may vary in their V antigen content.

E. This invention is novel because it is a single constructed protein composed of two unique proteins, the entire F1 capsule antigen and V antigen. It induces an immunological response against both the F1 protein and V antigen. It is also novel because it includes 2 protective immunogens in the same vaccine.

TABLE 1 Efficacy of F1-V vaccination against a lethal subcutaneous *Y. pestis* infection of mice

Treatment Group[a]	Strain	LD_{50}[b]	Survivors/Total
Alhydrogel alone	C12	57	0/10
13.6 µg F1-V	"	"	10/10
Alhydrogel alone	"	$1.1x10^6$	0/10
10 µg V	"	"	9/10
13.6 µg F1-V	"	"	9/10
27.2 µg F1-V	"	"	10/10

[a] For all groups, 8-10 week old female Swiss Webster (Hsd:ND4) mice (Harlan Sprague Dawley) were immunized subcutaneously on day 0 and day 28 with 0.2 ml of the indicated vaccine preparation. The F1-V and V proteins were each separately adsorbed to Alhydrogel, 1.3% (aluminum hydroxide gel adjuvant, Superfos Biosector).

[b] Mice were challenged with the $F1^-$, C12 strain, prepared as previously described (Welkos et al. Contrib. Microbiol. Immunol. 13:298-305, 1995), at day 78 after the initial antigen administration.

TABLE 2. Efficacy of F1-V vaccination against a lethal aerosol *Y. pestis* infection of mice

Treatment Group[a]	Strain	LD_{50}[b]	Survivors/Total	Geometric mean antibody titer[c] F1	V
Alhydrogel alone	C12	91	0/9	NT[d]	NT
13.6 μg F1-V	"	"	10/10	NT	NT
Alhydrogel alone	"	545-636	0/14	<640	<640
10 μg V[e]	"	545-636	8/10	NT	655,360
13.6 μg F1-V	"	545-636	10/10	66,540	432,376
27.2 μg F1-V	"	545-636	10/10	108,094	432,376
Plague USP[f]	"	545-636	0/8	55,738	<640
Alhydrogel alone	CO92	761	1/10	NT	NT
13.6 μg F1-V	CO92	761	10/10	NT	NT

[a]For all groups, 8-10 week old female Swiss Webster (Hsd:ND4) mice were immunized subcutaneously on day 0 and day 28 with 0.2 ml of the indicated vaccine preparation.

[b]Mice were challenged with inocula prepared as described in Table 1 at day 78 after the initial antigen administration. Aerosol exposures were performed in a nose-only exposure chamber with a dynamic small-particle aerosol as previously described (Welkos et al. Contrib. Microbiol. Immunol. 13:298-305, 1995). The apparatus was configured to challenge a maximum of 27 mice per exposure. Mice from several groups were divided between exposure runs to minimize differences among the treatment groups resulting in a dose challenge range.

[c]Serum obtained on day 58 after the initial immunization was assayed for anti-F1 and anti-V IgG antibody by ELISA on individual animals and group geometric mean titers determined. Titers were determined as the reciprocal of the maximum dilution giving an absorbance greater than 0.1 units after subtraction of nonspecific binding in normal serum.

[d]Not tested.

[e]Because F1-V was exposed to urea during purification, we also exposed this preparation of V to urea. V in PBS was buffer exchanged into 1x Binding Buffer

(Novagen) containing 6 M urea and placed at 4°C for 4 h after which urea was removed by dialysis as indicated for F1-V. V concentration was then determined and V was adsorbed to Alhydrogel.

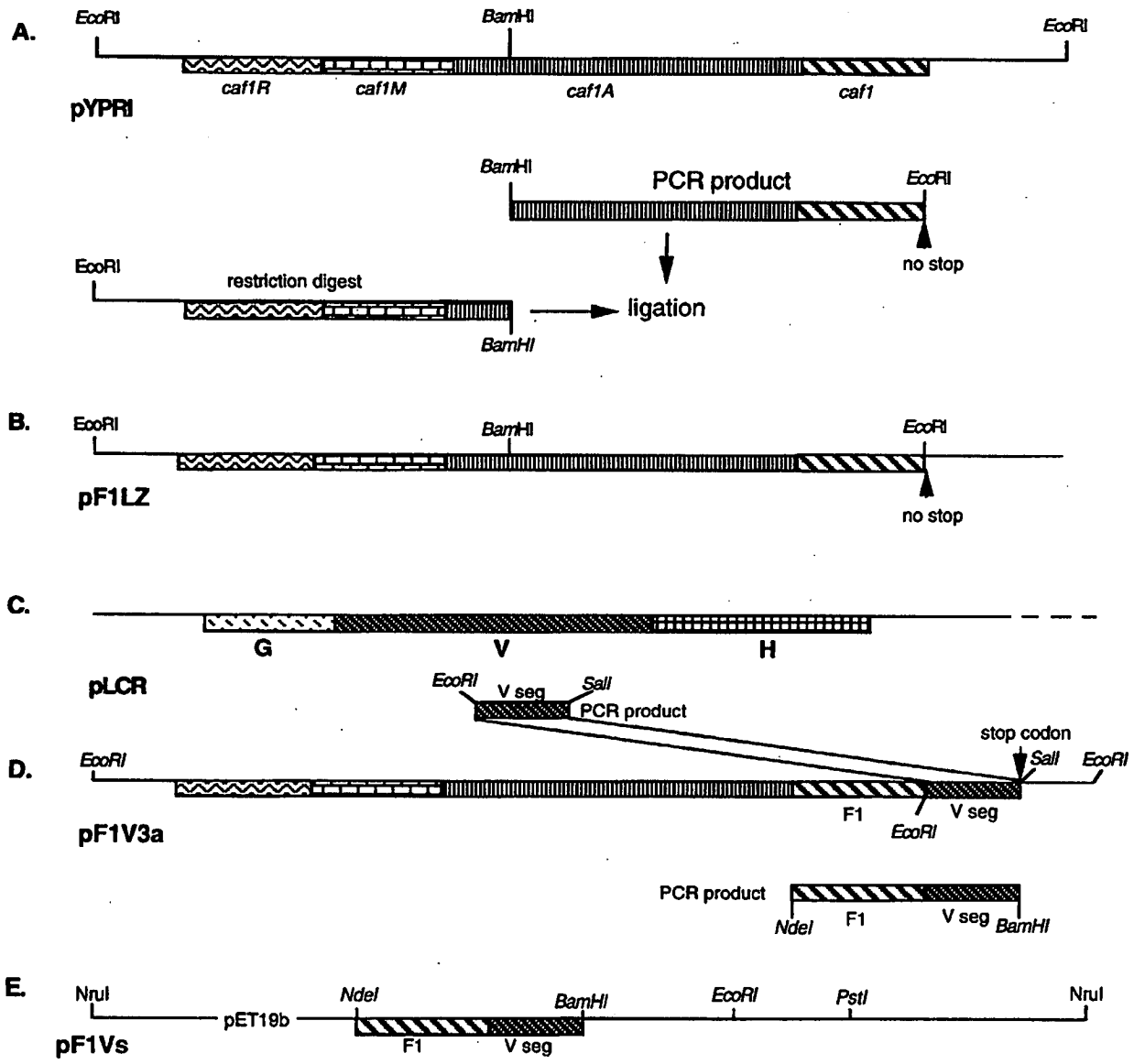
[f]The licensed, human, whole-cell plague vaccine United States Pharmacopeia (USP) was obtained from Greer Laboratories (Lenoir, NC).

TABLE 3. Survival of outbred mice after a single subcutaneous immunization followed by subcutaneous challenge.

Treatment Group[a]	Strain	LD_{50}[b]	Survivors/Total
Alhydrogel alone	CO92	5,750	0/10
Plague USP (Greer)	"	"	4/10
60.0 µg F1-V	"	"	10/10
Alhydrogel alone	C12	16,300	0/10
Plague USP (Greer)	"	"	0/10
60.0 µg F1-V	"	"	10/10

[a]For all groups, Hsd:ND4 Swiss Webster female 8-9 week old mice were immunized subcutaneously on day 0 with 0.2 ml of the vaccine preparation.
[b]Challenge was at day 44 postimmunization.

FIGURE 1.


A.
EcoRI
BamHI
EcoRI
caf1R
caf1M
caf1A
caf1
pYPRI
BamHI
PCR product
EcoRI
no stop
EcoRI
restriction digest
ligation
BamHI
B.
EcoRI
BamHI
EcoRI
pF1LZ
no stop
C.
G
V
H
pLCR
EcoRI
V seg
SalI
PCR product
stop codon
D.
EcoRI
SalI
EcoRI
F1
V seg
EcoRI
pF1V3a
PCR product
NdeI
F1
V seg
BamHI
E.
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EcoRI
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NruI
pET19b
F1
V seg
pF1Vs

RGURE 2

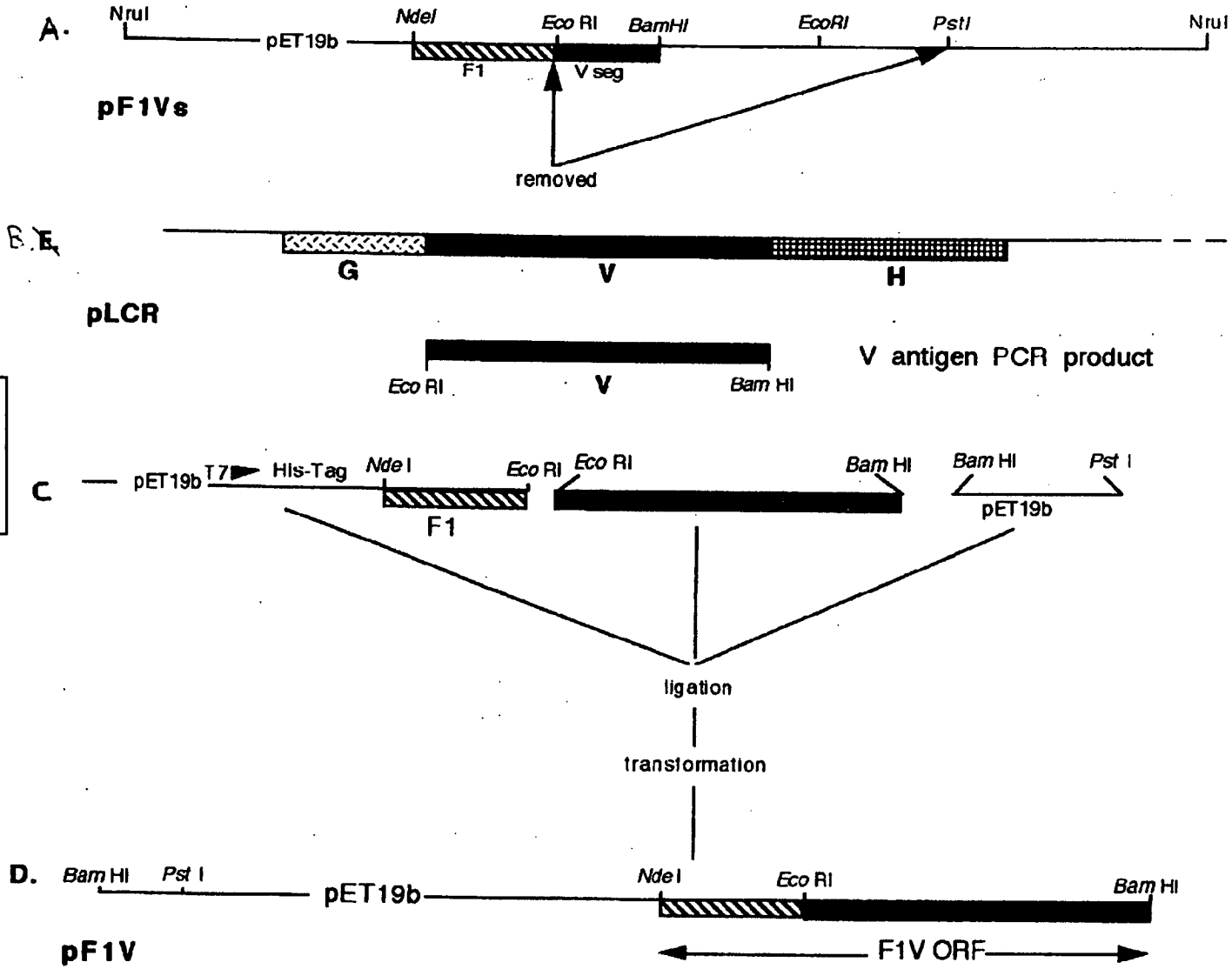
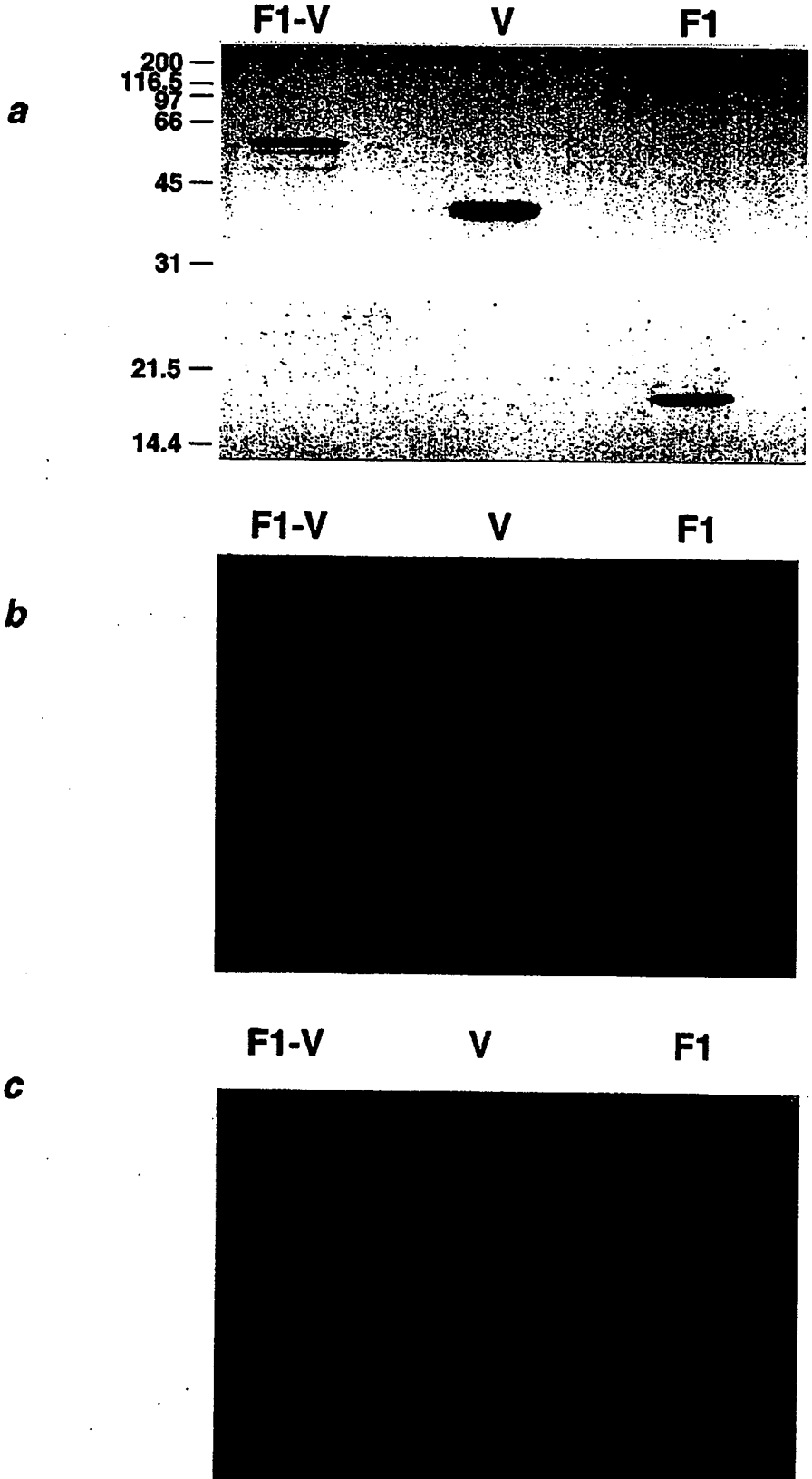


FIG. 3



CURRICULUM VITAE

Arthur M. Friedlander, M.D.

Birthplace: Brooklyn, New York

Education: Harvard College, A.B., 1961

University of Pittsburgh, M.D., 1965

Academic and Other Professional Positions:

7/65-6/66	Medical Intern, Downstate Medical Center, SUNY, Brooklyn, New York
7/66-6/67	Medical Resident, Downstate Medical Center, SUNY, Brooklyn, New York
7/67-6/69	Research Associate, National Cancer Institute, NIH, Bethesda, MD
7/69-6/72	NIH Postdoctoral Research Fellow, Infectious Diseases, University of California School of Medicine, San Diego, CA (Abraham I. Braude, M.D., Ph.D.)
7/71-6/72	Clinical Instructor of Medicine and Research, University of California School of Medicine, San Diego, CA
7/72-6/79	Assistant Professor of Medicine, University of California School of Medicine, San Diego, CA
7/79-7/86	Principal Research Investigator, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD
8/86-12/88	Chief, Airborne Diseases Division, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD
1/89-6/90	Chief, Department of Pathobiology, Pathology Division, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD
7/90-7/98	Chief, Bacteriology Division, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD
8/98-1/02	Senior Military Scientist, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD
2/02-	Senior Scientist, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD

Clinical, Teaching, and Administrative Positions:

Lecturer and Laboratory Instructor, Medical Microbiology Course, University of California School of Medicine, San Diego, CA, 1972-1979
Attending Physician, Internal Medicine, University of California Medical Center, San Diego, CA, 1972-1979
Attending Physician, Infectious Diseases, University of California Medical Center, San Diego, CA, 1972-1979
Chairman, Hospital Infection Control Committee, University of California Medical Center, San Diego, CA, 1976-1978
Attending Physician, Infectious Diseases, Walter Reed Army Medical Center, Washington, D.C., 1980, 1982, 1984-1991
Clinical Associate Professor of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, 1991-1998
Adjunct Professor of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, 1998-present
Director, AMEDD Course on Medical Defense Against BW and Infectious Diseases, 1984-1991
Lecturer, AMEDD Course on Medical Defense Against BW and Infectious Diseases, 1980-1995
Chairman, Human Scientific Review Committee, USAMRIID, Frederick, MD, 1990-2002
Member, Infectious Diseases Society of America Committee on Emerging Infections and Bio-Defense

Medical Licensure: Maryland

Military Service: LCDR, USPHS, National Institutes of Health, Bethesda, MD, 7/67-7/69
MAJ, U. S. Army, MC, USAMRIID, Frederick, MD, 7/79-7/80
LTC, U. S. Army, MC, USAMRIID, Frederick, MD, 7/80-7/86
COL, U. S. Army, MC, USAMRIID, Frederick, MD, 7/86-3/02

Honors and Fellowships:
Nerve-Muscle Research Fellow (Stephen Kuffler), Marine Biological Laboratories, Woods Hole, MA 6/64-9/64
Alpha Omega Alpha (Medical School Honor Society)
NIH (NIAID) Postdoctoral Fellowship, 7/69-7/71
Fellow, Infectious Diseases Society of America
Army Research & Development Achievement Award
Order of Military Merit
Jay P. Sanford Memorial Award for Excellence in Military Infectious Diseases from the Armed Forces Infectious Diseases Society (2005)

Patents Filed
1. Development of an attenuated strain of *Bacillus anthracis* for production of a recombinant anthrax vaccine.
2. Method of making a vaccine for anthrax.

3. An improved recombinant F1-V fusion protein vaccine against plague.
4. Polyglutamic acid depolymerase therapeutic for anthrax.

Other

Testimony before House Committee on Government Reform Hearing on Anthrax Vaccine Immunization Program: 3 Oct 2000
Testimony before Senate Intelligence Committee: 25 Oct 2001
Testimony before House Intelligence Committee: 31 Oct 2001
Testimony before House Committee on Government Reform: 28 Feb 2002

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